

# The effects of allopurinol on rat liver and spleen tissues in a chronic hyperammonemia animal model

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## ABSTRACT

**Objectives:** To investigate whether hyperammonemia can lead to any structural change in liver and spleen tissues or biochemical changes in blood and if allopurinol (ALLO) has a protective effect in hyperammonemia.

**Methods:** This study was conducted between April and May 2006. Thirty-six females Wistar Albino rats were randomly divided into 3 equal groups: Controls, administered with ammonia (NH<sub>3</sub>) and administered with NH<sub>3</sub> + ALLO groups. Ammonium acetate (2.5 mmole/kg/day) was injected to NH<sub>3</sub> group intraperitoneally (IP) for 28 days. The other group received ammonium acetate (2.5 mmole/kg) plus ALLO (50 mg/kg) IP for 28 days. After finishing the study, blood and tissue samples were collected to perform histopathological and biochemical analysis.

**Results:** Liver and spleen tissues were normal in the control group. In NH<sub>3</sub> group, liver tissues were minimally vacuolar and granular degenerations and moderate mononuclear cell infiltration. However, there was no histopathological change in NH<sub>3</sub> + ALLO group. Spleen tissues were normal in NH<sub>3</sub> group. In biochemical analysis, there was no significant difference between the groups ( $p>0.05$ ).

**Conclusion:** The ammonium acetate may cause minimal structural changes in rat liver and ALLO can prevent this. We found that biochemical parameters do not necessarily correlate with the histopathological findings.

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Ammonia (NH<sub>3</sub>) is present in body fluids and it is converted to urea in liver through the urea cycle mechanism. Ammonia shows its toxic effect mainly on central nervous system. Increased NH<sub>3</sub> exposure in brain tissue is the primary cause of neurological disorders. Hyperammonemia is associated with some congenital urea cycle enzyme disorders, hepatic encephalopathy syndrome, other metabolic disorders and toxic encephalopathies. As NH<sub>3</sub> is detoxified in liver, hepatic encephalopathy (HE) is an important complication of liver cirrhosis. Although the importance of neurotoxins and especially NH<sub>3</sub> has been emphasized, the underlying mechanisms in pathophysiology are not completely understood.<sup>1</sup> Etiology of HE is explained by a multifactorial theory. On the other hand, NH<sub>3</sub> accumulation has traditionally been considered to have the most important role on the pathogenesis, although there are so many compounds that accumulate in the circulation as a consequence of impaired liver function.<sup>2,3</sup> Possible pathogenic mechanisms were extensively investigated using some special animal models or cultures of the central nervous system cells treated with neuroactive substances that have been implicated in HE. The availability of an animal model is crucial to study on the pathophysiological mechanisms and to test the possible therapies in HE. There are several animal models for this kind of research, but none of them are enough to be a sufficient model for chronic liver disease accompanying HE and abnormalities in nitrogen metabolism as seen in humans.<sup>4,5</sup> Two animal models are widely used: Portocaval anastomosis operation and chronic hyperammonemia model.<sup>6</sup> In the latter, normal animals are fed with ammonium acetate supplemented diet and hyperammonemia is induced.<sup>7</sup> Ammonia can also be administered parenterally.<sup>8-12</sup> There are numerous studies reporting that xanthine oxidase (XO) may contribute to some changes in tissues during various pathological conditions via free radical mechanism. Reactive oxygen species generated in enzymatic process is involved in oxidative damage.

Thus, the inhibition of this enzymatic pathway would be beneficial.<sup>13-18</sup> In this study, we aimed to investigate whether hyperammonemia causes structural changes in liver and spleen tissues or biochemical alterations in blood parameters and whether allopurinol (ALLO) has any protective effect against hyperammonemia toxicity.

**Methods.** Thirty-six females 10-14 weeks old Wistar Albino rats weighting between 160 and 200 grams were obtained with specific-pathogen-free status from Suleyman Demirel University, Faculty of Medicine, Animal Laboratory, Isparta, Turkey, between April and May 2006. All animals were kept under standard laboratory conditions,<sup>19</sup> filtrated municipal tap water (Isparta, Turkey) and standard laboratory rat feed (Abalioglu Yem Sanayii, Denizli, Turkey) was provided ad libitum. Feed and water consumption were followed daily after the acclimatization period. Rats were randomly divided into 3 groups as follows: Controls (n=12), administered with NH<sub>3</sub> group (n=12) and administered with NH<sub>3</sub> + ALLO group (n=12). Ammonium acetate and phosphate buffered saline (PBS) were obtained from Sigma Chemical Co, St. Louis, MO, USA. Allopurinol was obtained from Ilsan-Iltas Chemical, Turkey. Briefly, weights were measured immediately before the experiment and a PBS solution was injected intraperitoneally (IP) to the controls. To determine the dose range, we referred to a study conducted by Hermenegildo et al.<sup>11</sup> They reported that dose-dependent acute exposure to NH<sub>3</sub> is the variable so that, injection of 3 mmol/kg or less of NH<sub>3</sub> has no neurological symptoms, whereas 3.5-4.5 mmol/kg of NH<sub>3</sub> caused precoma and 5-5.5 mmol/kg caused death in animals.<sup>11</sup> We performed a preliminary study to determine the accurate dose range in chronic NH<sub>3</sub> exposure. As ammonium acetate (2.5 mmole/kg/day) was injected IP to ammonia group for 28 days, ammonia plus allopurinol group has received ammonium acetate (2.5 mmole/kg) and allopurinol (50 mg/kg) (IP). Chemicals were dissolved in PBS, and the pH was adjusted and the solutions were injected IP using an injection filter. The injection volume was 1.0 ml for 200 grams body weight. For each study day, this application was performed at 9:00 a.m. After 28 days, the study has been finished. All animals have undergone sternal dissection process under ether anesthesia and blood samples were taken directly from the heart. Spleen, liver and other organs were picked for histopathological examination. Splenic and hepatic tissues were fixated in 10% formaline solution. After histopathological tissue processing procedure, tissue samples were embedded in paraffin blocks.

**Histological analysis.** For light microscopic evaluation, 15 sections were taken from each block

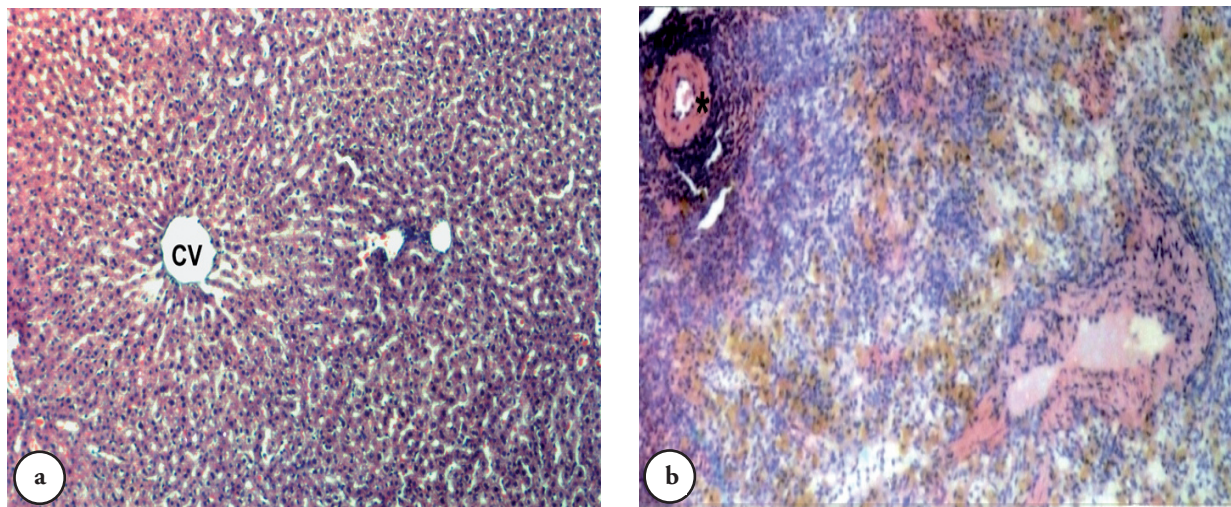
using systematical randomized sampling method. All sections were stained with hematoxylin - eosin dye. Stained sections were examined under Olympus - BX 50 light microscope. The portal area diameter was calculated as the mean diameter  $[(t_r + v_r) / 2]$  from the vertical ( $v_r$ ) and transverse ( $t_r$ ) diameters measured by an ocular micrometer for 100 portal areas with 10 fold magnification. The mean  $\pm$ SD sinusoidal width has an average of 1000 measurements.<sup>20</sup> The method of Tsukamoto et al<sup>21</sup> was modified to evaluate the hepatocyte degeneration, as follows: 1+= 0-25% of hepatocytes with degeneration (little); 2+= 25-50% (median); 3+= 50-75% (high), and 4+= >75% (very high) (**Table 4**). Splenic white pulp and central artery diameters were measured as the portal triad diameter.

**Biochemical analysis.** Biochemically, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) levels were measured from blood samples to evaluate the liver functionality. An autoanalyser (Abbott Aeroset, Abbott Park III, USA) was used.

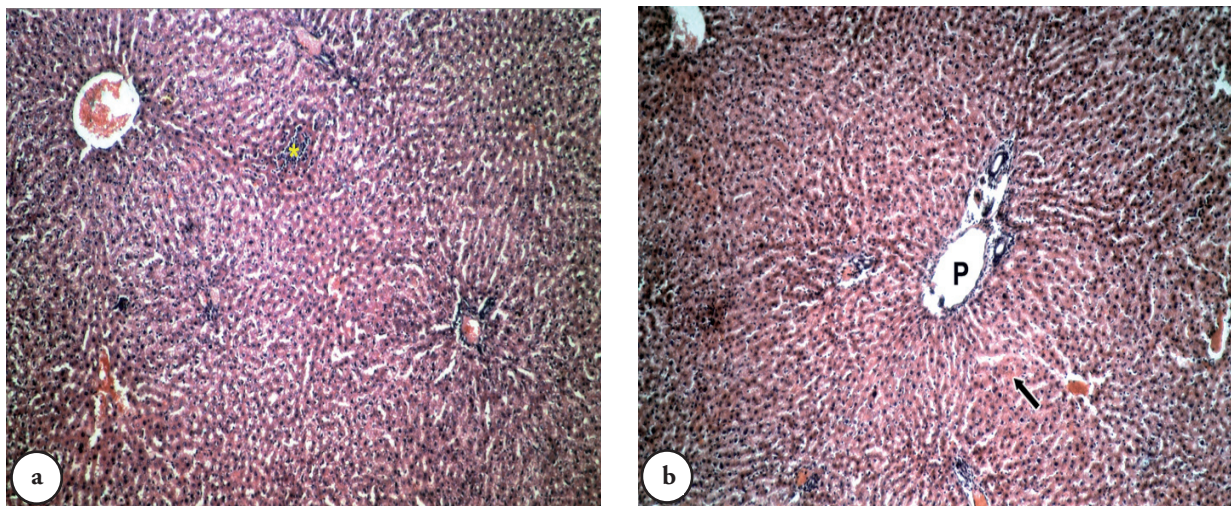
Statistical Package for Social Sciences Version 11.0 was used for statistical analysis. All data were presented as mean value  $\pm$  standard deviation (SD). Statistical analysis was performed with Student's t-test. Only hepatocyte count parameter was evaluated via using Chi-square test. The  $p$  values under 0.05 were considered as statistically significant.

**Results.** There was no histopathological change in liver and spleen tissues of the control group (**Figure 1a and 1b**). Whereas, in liver tissues of the NH<sub>3</sub> group, there were minimal vacuolar and granular degenerations and moderate mononuclear cell infiltration near the portal triad area (**Figure 2a**) (**Table 1**). No histopathological changes was seen in NH<sub>3</sub> + ALLO group (**Figure 2b**) (**Table 1**). We have measured the portal triad, sinusoidal width, and central vein diameters (**Table 2**). There was no meaningful difference among 3 groups. In histopathological examination of the spleen tissue, white pulp, and central artery diameters were evaluated. Also, there was no statistically significant difference between the controls and NH<sub>3</sub> group (**Figure 3a and 3b**) (**Table 3**). But as mentioned above, observed minimal degeneration is not necessarily supported by the biochemical evidence. In biochemical analysis, there was no statistically significant difference between the groups by means of AST, ALT, and LDH values ( $p > 0,05$ ). The biochemical parameters were given in **Table 4**.

**Discussion.** Ammonia is a compound of normal body fluids. The mean concentration is 35 mmole/lit. Excessive NH<sub>3</sub> is converted to urea through urea cycle in the liver. Bacterial urea hydrolysis, purin nucleotide



**Figure 1** - Histopathological changes **a**) normal liver tissue from the control group (hematoxyline & eosin, x10), **b**) normal spleen tissue from the control group (Starred: central artery) (hematoxyline & eosin, x10). CV - central vein



**Figure 2** - Histopathological changes **a**) moderate cell infiltration (starred) in only NH<sub>3</sub> group (hematoxyline & eosin, x10), **b**) minimal degeneration (arrow) in only ammonia (NH<sub>3</sub>) and NH<sub>3</sub> + allopurinol administrated groups (hematoxyline & eosin, x10). P - portal area

**Table 1** - The effects of ammonia (NH<sub>3</sub>) administration to rats with or without allopurinol (ALLO) in liver tissues ( $p>0.05$ ).

Parameters	Controls (n=12)		NH <sub>3</sub> (n=12)		NH <sub>3</sub> + ALLO (n=12)	
	ND	SD	ND	SD	ND	SD
Hepatocyte degeneration	98	2	87	13	96	4

ND - no degeneration, SD - small degeneration

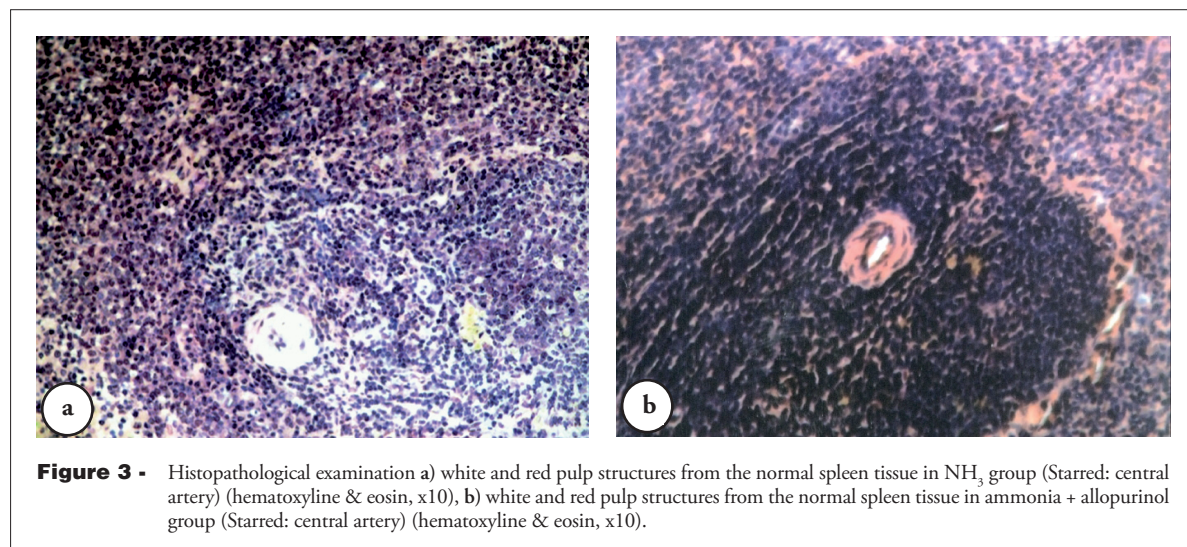
**Table 2** - The effects of ammonia (NH<sub>3</sub>) and NH<sub>3</sub> + allopurinol (ALLO) administration on liver tissues of rats. (Measurement unit: U/l).

Parameters	Controls (n=12)	NH <sub>3</sub> (n=12)	NH <sub>3</sub> + ALLO (n=12)
Portal triad diameter	19.3 ± 9.42	22.13 ± 9.59	20.91 ± 8.05
Sinusoidal width	3.91 ± 0.79	4.14 ± 0.99	3.41 ± 0.51
Central vein diameter	21.91 ± 12.66	25 ± 13.13	24.5 ± 10.5

The  $p>0.05$  for all the groups.

cycle and amino acid transamination process in skeletal muscles and other metabolic activities of liver and kidneys are the intrinsic sources of  $\text{NH}_3$  and this  $\text{NH}_3$  production does not cause any pathological situation under normal conditions. However, when this cycle is broken due to some disorders such as acute liver failure or dysfunction, then hyperammonemia could occur and this leads to some undesirable insults. The most common complication of hyperammonemia is HE.<sup>22</sup> Despite the numerous studies regarding HE, there is no consensus on how it occurs. Recently, some possible mechanism has been postulated.<sup>3</sup> The main idea in this controversy is focused on  $\text{NH}_3$  intoxication.<sup>2,9,23-26</sup> Some animal models were developed to test the possible responsible factors. We have observed that hyperammonemia causes minimal structural change in liver tissue. This evidence suggests multiple factors including XO. Serum XO levels significantly increase in some conditions such as hepatitis, liver inflammation, ischemia/reperfusion model, and carcinogenesis, XO

causes damage by increasing free radical amount in tissues.<sup>13-18,27,28</sup> Free radical-induced changes lead to more XO release from tissues. It has been reported that purin metabolites and acetaldehyde are converted to their substances by XO. This process also increases the free radical amount in liver tissue.<sup>29</sup> However, it has been mentioned that XO inhibition has a protective effect against free radical mediated damage.<sup>30</sup> In our study, we have observed mononuclear cell infiltration in  $\text{NH}_3$  group. Polymorph nuclear cell-endothelium adherence is, partly, a response to reactive oxygen radicals, as well as chemotactic cytokines.<sup>31</sup> This adherence results in microvascular failure, frequently called "no reflow phenomenon" and tissue injury.<sup>32,33</sup> The ALT, AST, alkaline phosphatase, glutamyl transpeptidase and LDH are the most common evaluated enzyme parameters in liver studies. Although these enzymes are not specific, they are regarded as effective markers of hepatic destruction. Increased serum AST and ALT levels in  $\text{NH}_3$  group suggest liver tissue impairment.<sup>34-36</sup> In one



**Figure 3** - Histopathological examination a) white and red pulp structures from the normal spleen tissue in  $\text{NH}_3$  group (Starred: central artery) (hematoxyline & eosin, x10), b) white and red pulp structures from the normal spleen tissue in ammonia + allopurinol group (Starred: central artery) (hematoxyline & eosin, x10).

**Table 3** - The effects of ammonia ( $\text{NH}_3$ ) and  $\text{NH}_3$  + allopurinol (ALLO) administration on spleen tissue of rats (measurement unit: U/l).

Parameters	Controls (n=12)	$\text{NH}_3$ (n=12)	$\text{NH}_3$ + ALLO (n=12)
White pulp diameter	24.32 ± 5.23	27.13 ± 7.18	25.46 ± 5.67
Central artery diameter	10.37 ± 4.27	12.33 ± 4.13	11.06 ± 6.21

The  $p > 0.05$  for all the groups.

**Table 4** - The effects of ammonia ( $\text{NH}_3$ ) administration with or without allopurinol (ALLO) (measurement unit: U/l).

Parameters	Controls (n=12)	$\text{NH}_3$ (n=12)	$\text{NH}_3$ + ALLO (n=12)
AST	156.36 ± 39.83	174.45 ± 25.43	167.42 ± 36.23
ALT	62.90 ± 16.15	63.81 ± 8.13	54.85 ± 19.54
LDH	1843.09 ± 416	2273 ± 948.18	2117 ± 178.81

AST - aspartate aminotransferase, ALT - aminotransferase, LDH - lactate dehydrogenase, the  $p > 0.05$  for all the groups.

study, Holecsek et al<sup>37</sup> found that as ammonium infusion decreased the protein synthesis in skeletal muscles, it did not affect the other organs such as liver, spleen, intestines or kidneys in the same degree.

In the present study, although there was a minimal structural changes in the liver, no change was observed in serum AST and ALT levels. Low NH<sub>3</sub> dose can lead to this condition. Minimal changes can prevent by antioxidant defence systems and there may be no change in biochemical parameters. It has been reported that acute liver failure was associated with accelerated glycolysis and lactate increment in splanchnic region without hypoxia.<sup>38-40</sup> Although our study was based on a chronic-term administration process, lack of structural changes in spleen tissue supports this hypothesis. It has been reported that ALLO decreased the amount of free radicals in ischemic intestine tissue.<sup>6</sup> In another study, it has been found that ALLO inhibits the formation of reactive oxygen species to prevent liver damage.<sup>41-44</sup> This agent also induces the adenosine triphosphate (ATP) and phosphoenolpyruvate carboxykinase (GTP) synthesis.<sup>45,46</sup> Lack of minimal hepatic degeneration and decrement in mononuclear cell infiltration in NH<sub>3</sub> + ALLO group<sup>31</sup> may relate to the inhibition of XO enzyme activity, free radical formation, induction of ATP and GTP synthesis by ALLO.

In conclusion, the ammonium acetate given at a dose of 2.5 mmole/kg for 28 days causes minimal structural changes in rat liver and ALLO can prevent this. Also, we observed that biochemical parameters do not necessarily accompany with the histopathological findings.

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Acquisition of funding, collection of data, or general supervision of the research group, alone, does not justify authorship.

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